Exosomes as Liquid Biopsy Biomarkers in Diffuse Large B-cell Lymphoma (DLBCL)- Current State-of-the-Art and Unmet Clinical Needs

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Abstract

Diffuse Large B-Cell Lymphoma is the most common type of Non-Hodgkin's Lymphoma. The disease exhibits significant clinical and biologic heterogeneity. Treatment with standard first line therapy results in cure in about 60% of patients while 30-40%of patients either are refractory to therapy or relapse. Current prognostic scores and biomarkers are unable to accurately predict patients who would relapse or would have refractory disease. A part of the heterogeneity in the behavior of DLBCL is explained by the cell of origin of the tumor. Germinal center type (GCB) DLBCL which is derived from centroblasts are associated with better prognosis compared with activated B-cell type (ABC), which is derived from a B-cell committed to secretory differentiation. While the gold standard for cell of origin determination is gene expression profiling, immunohistochemical methods are routinely used because of more readily available fixed tissue and expertise. Immunohistochemical methods are however associated with a significant degree of discordance with GEP. Within the ABC and GCB types of DLBCL, subgroups of prognostic significance have been identified using various multiple approaches which do not inure themselves to routine practice partly because of limitation of diagnostic material or expertise. Exosomes are a class of membrane bound extracellular vesicles of endosomal origin, produced by multiple cell types. They are involved in intercellular communication and present in abundance in various bodily fluids. Exosomal cargo which includes nucleic acids and proteins can be analyzed, yielding diagnostic and prognostic information in management of DLBCL.

Introduction

Diffuse large B-Cell lymphoma (DLBCL) is the most common type of Non Hodgkin Lymphoma (NHL) constituting 30-40% of cases (1,2,3). An estimated 27,650 new cases were diagnosed in 2016 with 25,380 of them being the not otherwise specified (NOS) variant (4). DLBCL shows a slight male predominance (55%) and the median age at diagnosis is 64 years, but the disease affects all age groups and age of onset is earlier in African Americans (5,6). The 5-year survival rate shows mild geographic variation, 62% in the US (7) and 55.4% in Europe (8).

DLBCL, despite its relative morphologic homogeneity, represents heterogeneous entities, with disparate biological and clinical manifestations. Outcome of disease to standard first line therapy, R-CHOP, consisting of rituximab, cyclophosphamide, doxorubicin, vincristine, and prednisone plus the monoclonal antibody rituximab, is variable. Twenty to fifty percent of patients are either refractory to therapy without remission or relapse after complete remission (9,10,11)

Relapsed disease is associated with a poor prognosis and patients with early disease relapse have a worse prognosis compared to those with late relapses (12). The mechanisms for relapse are purportedly different for these two groups (13). The prognosis for refractory patients, patients whose disease fails to go into

remission or progress with induction therapy, is even more dismal compared to early and late relapse disease (14). While patients with relapse and refractory disease might have had need for more aggressive first line therapies, there are currently no biomarkers that adequately predict at diagnosis, the patients who are likely to be refractory to therapy, relapse after initial remission, or the time of relapse whether early or late. Similarly, once treatment commences or remission is achieved, there are no biomarkers that can accurately predict subsequent behavior of the disease. In addition, in refractory and relapsed disease, biomarkers capable of predicting response to second or additional lines of therapy remain elusive. Thus, there exists an urgent, unmet clinical need for biomarkers that address these challenges in management of DLBCL.

Current disease stratification systems/methods and assays

International Prognostic Index for Risk Stratification of DLBCL patients

The International Prognostic Index (IPI) and its revised variant, R-IPI are widely used clinical prognostication systems for DLBCL (15,16). These indices use a combination of age, Eastern Cooperative Oncology Group (ECOG) performance status, tumor stage, lactate dehydrogenase level, and the number of extra nodal sites of disease to predict the prognosis of patients with DLBCL (17). Biccler et al showed the poor performance of the IPI in predicting overall survival in DLBCL patients. (18) In a study investigating the ability of the IPI and the revised index to predict outcome, R-IPI identified three distinct prognostic groups with: (a) very good (4-year progression-free survival [PFS] 94%, overall survival [OS] 94%), (b) good (4-year PFS 80%, OS 79%), and (c) poor (4-year PFS 53%, OS 55%) outcomes, respectively ($P< 0.001$) (19). However, neither the R-IPI nor IPI are unable to predict patients with <50% chance of survival. Such patients potentially need more potent first lines of therapy. However, there are no biomarkers that can reliably identify these high risk patients as of yet.

Cell of Origin (COO) determination

The cell of origin of DLBCL explains part of the heterogeneity of the disease. Using gene expression profiling, DLBCL NOS was initially classified into germinal center B cell (GCB), activated B-cell (ABC), and non-classifiable types (20). GCB type DLBCL is thought to derive from centroblasts while the ABC type has features reminiscent of a B-cell committed to terminal B-cell differentiation (21) (see Figure 1).

The COO classification could predict overall survival of DLBCL patients and response to R-CHOP therapy. The GCB subtype has a more favorable prognosis compared to the ABC subtype. (22,23,24,25). Determination of cell of origin also has implications on drug therapy in relapsed/refractory disease. Recently, ibrutinib, a BCR inhibitor was found to be effective in relapsed and refractory ABC type DLBCL (26).

The gold standard method for determining COO has been gene expression profiling (GEP) (27). This modality, however, is not available at all centers and many assays require fresh tissue, which may not always be available. In routine practice, various immunohistochemistry (IHC) based algorithms (see Figure 2) such as Hans, Tally, Choi and Visco-Young are used to determine COO for DLBCL (28,29,30,31). IHC methods for COO assignment are more practical and widely available, but are plagued by inter-observer variability that can result in discordant classification compared with the gold standard. Gutiérrez-García et al demonstrated that when compared with GEP, different IHC algorithms; Colomo, Hans, Muris, Choi, and Tally, misclassified cases at a higher rate when defining the GCB subset: 41%, 48%, 30%, 60%, and 40%, respectively (32). Gene-expression profiling and not immunophenotypic algorithms predicts prognosis in patients with diffuse large B-cell lymphoma treated with immunochemotherapy. In this study, while the GEP-defined groups showed significantly different 5-year progression-free survival (76% vs 31% for GCB- and ABC- DLBCL) and overall survival (80% vs 45%), none of the IHC algorithms retained the prognostic impact of the COO groups (GCB vs non-GCB). Other studies have also suggested that IHC algorithms may not have the same prognostic impact as other methods of COO determination (33,34,35). These results underscore the unmet clinical need for a methodology that uses readily available biopsy material for accurate COO classification, concordant with GEP while maintaining prognostic utility.

The COO classification is not without limitations however. COO does not explain all the heterogeneity in

the behavior and prognosis of DLBCL. Using various approaches, subgroups within ABC showing favorable prognosis have been identified and adverse prognostic groups have also been identified for the GCB type. In a study involving 574 DLBCL biopsy samples using exome and transcriptome sequencing, array-based DNA copy-number analysis, and targeted amplicon resequencing of 372 genes to identify genes with recurrent aberrations, Schmitz et al. identified four DLBCL genetic subtypes: BN2, EZB, N1, and MCD, with the former two determined to have favorable prognosis and the latter two associated with poor prognosis (36). Progression free survival and overall survival varied significantly within these groups. Of note, heterogeneity in behavior within the ABC group could be identified, with inferior survival in the MCD and N1 subtypes of ABC and favorable survival in the BN2 subtype. In GCBs, EZB subtype had a worse predicted 5-year survival compared with other GCBs non-classifiable by this system. Significant independent and additive contributions to survival by gene expression profiling (ABC vs GCB) were noted in this genetic subtyping model.

Chapuy et al used a combination of recurrent mutations, somatic copy number alterations

and structural variants (SV) to identify five distinct groups of DLBCL including a hitherto unappreciated group of ABC-DLBCL with favorable prognosis (37). They also identified a subtype of GCB-DLBCL with poor prognosis characterized by mutations and structural variants of*BCL2,* mutations in *PTEN* and chromatin modifiers such as*KMT2D* , *CREBBP* , and *EZH2* and focal*10q23.31/PTEN* loss. These subgroups within the different COO types provide insight not only for prognosis but also suggest possible mechanisms for therapeutic intervention. In spite of the usefulness of these multiparametric approaches for classification, it will be challenging to apply these genetic classification models in the clinical settings due to limitations in the amount of diagnostic material available, resources and bioinformatics expertise.

Disease Monitoring During Therapy

The rationale of using multiple agent chemotherapies in the treatment of cancers is to avoid development of resistance, but resistance does develop regardless of the type and combination of therapy. The neoplastic cells may develop adaptive resistance to chemotherapy in the course of treatment. Serial tumor profiling can provide insight into the pathways mediating adaptive resistance and identify targets for novel therapeutic drug development to overcome the resistance mechanisms (38). Real time analysis may also allow prompt detection of resistance and suggest alteration of therapy if signatures of resistance portending refractoriness or relapse are detected. In DLBCL patients, it is not usually feasible to obtain tissue regularly for such profiling without invasive measures, which cause patient discomfort and increase the risk of infections from multiple biopsy procedures. Thus, a non-invasive method for tissue sampling at multiple time points would be of great benefit.

Post Treatment Surveillance

The role of post-therapy or remission imaging surveillance in the management of DLBCL patients is controversial. While the National Comprehensive Cancer Network (NCCN) and European Society of Medical Oncology (ESMO) guidelines recommend surveillance imaging for stage III/IV disease for the first two years after completion of front-line therapy (39), the 2014 Lugano classification system advises against routine surveillance imaging (40). The positive predictive value of post treatment PET is low, resulting in patient anxiety and increased costs for these unnecessary medical procedures (41,42). Studies have also failed to establish a survival advantage in imaging detected disease relapse (43,44). Realization of the lack of survival benefit of surveillance imaging has resulted in decreased rates of surveillance, although this practice is still quite frequent, with over half of DLBCL patients diagnosed in 2014-2016 undergoing surveillance imaging (45). Thus, there is a pressing need for more specific and sensitive methods for detecting recurrence of disease while also avoiding risks of radiation exposure (46).

Novel Methodologies in DLBCL Management- Liquid Biopsy Based Approaches

Liquid biopsy techniques which involve assessment of cancer related biomarkers in bodily fluid samples, have potential to resolve the unmet clinical needs in management of DLBCL. Common liquid biopsy techniques

include circulating tumor cells (CTC), circulating tumor DNA (ctDNA) and exosomes.

Circulating Tumor Cells

Circulating tumor cells are neoplastic cells that are passively shed or actively intravasate into circulation from primary tumors and/or metastatic deposits. (47). The number of CTCs in cancer patients correlate with treatment outcome and overall survival (48). The DNA, RNA and protein content of CTCs can be evaluated for molecular characterization informative of native tumor tissue (49). CTCs constitute a small fraction of cells in circulation-1 in 10^9 cells (50) and thus various techniques using distinct biological and physical properties of CTCs are used to differentiate and isolate them from leucocytes, erythrocytes and other cells in a blood sample. The predominant method of CTC isolation involves enrichment for cells with EPCAM expression and lack of CD45 expression (51,52,53). This is not applicable to circulating DLBCL cells and cells from other hematological malignancies which express CD45 and lack EpCAM expression. In addition, the low abundance of CTCs makes their use in early stage of disease challenging. Other challenges with use of CTC as liquid biopsy is the need for prompt processing of whole blood after collection in most isolation platforms (54) precluding the potential for biobanking specimens for future analyses or use of archival biobanked specimens for studies.

Cell free DNA and Cell tumor DNA

Non cell bound DNA fragments are shed into circulation as cell free DNA (cfDNA). Circulating tumor DNA (ctDNA) which is cfDNA from tumor cells is shed into blood through apoptosis, necrosis or through an active process from neoplastic cells. Molecular aberrations in tumor tissues such as point mutations, insertions, deletions and methylation profiles are present in these circulating DNA fragments (55,56,57,58,59). The fragmentation pattern of ctDNA in healthy people has been found to be different from the pattern in patients with various kinds of cancers (60). Surveillance circulating tumor DNA identifies patients at risk of recurrence in DLBCL with a positive predictive value of 88.2% and negative predictive value of 97·8% (61). Unlike CTCs and exosomes which contain RNA and proteins together with DNA, ctDNA provides no proteomic and transcriptomic information.

Immunoglobulin gene rearrangement is another novel technology for identifying prognostic groups in DLBC. Molecular characterization of immunoglobulin gene rearrangements revealed distinct subgroups in non-GCB (62). DLBCL patients with immunoglobulin gene rearrangement have been shown to have a lower rate of complete remission and significantly poorer survival. (63).

Exosomes

Exosomes are membrane-bound vesicles ranging from 30-120 nm in diameter, and are released upon fusion of multi-vesicular bodies with the cell membrane (64,65,66). The contents of exosomes include proteins, nucleic acids, lipids and sugars, and they are thought to be involved in intercellular communication (67,68,69,70,71). Exosomes are endosome derived extracellular vesicles. Early endosomes are formed from internal budding of the cell membrane with accompanying membrane proteins and lipids into the cytoplasm. As the endosome matures, there is transport of cytosolic, golgi and nuclear cargo such as nucleic acids, proteins and other metabolites into the lumen of the endosome. There is subsequently intravesicular budding of the endosomal membrane, forming multivesicular bodies (MVB). The MVB fuses with the cell membrane releasing its vesicles as exosomes into the extracellular space. Involvement of the endosomal pathway differentiates exosomes from microvesicles and apoptotic bodies which are the other extracellular vesicles. Microvesicles and apoptotic bodies are larger vesicles formed from outward budding of the cell membranes from living and dying cells respectively, into the extracellular space (72)

Mechanisms by which recipient cells take up exosomes include macropinocytosis, receptor or lipid raft mediated endocytosis, phagocytosis, or direct fusion with the recipient cell membrane (73). Exosomes are found in many body fluids including blood, urine, cerebrospinal fluid, amniotic fluid, saliva, breast milk, peritoneal fluid, etc. (74).

Exosome Isolation Methods

There are various methods for isolating exosomes with varying degrees of complexity and contamination with non-exosomal vesicles and/or non-vesicular products. The prevalent method for exosome isolation is differential ultracentrifugation. It involves sequential spinning of a fluid at increasing speed and duration to pellet dead cells and cellular debris, microvesicles and eventually, exosomes (75). This method is not suitable for fluids with high viscosity such as blood since higher speeds and longer duration of centrifugation are required (76). Due to similarity of sedimentation properties of various types of extracellular vesicles the yield and purity using this method may be low (77,78)

Density gradient ultracentrifugation uses a concept similar to differential ultracentrifugation. The initial steps are similar to DUC but sucrose is added at a later point. Due to the buoyant density of exosomes (1.15 to 1.19 g/mL) in sucrose, it sediments separately from other denser extracellular vesicles during centrifugation (79,80). This method yields purer fractions compared with differential ultracentrifugation (81), but has same disadvantage of need for long periods of processing and requirement of costly equipment.

Size based isolation techniques make use of the smaller size of exosomes compared with microvesicles, apoptotic bodies and other molecules to separate them in a fluid sample. Ultrafiltration, a size based technique uses membrane filters with decreasing pore sizes to isolate exosomes (82). It is a faster method compared with ultracentrifugation and does not require costly equipment. While this method yields pure vesicles, there is difficulty in removing contaminating proteins but can be combined with ultrcentrifugation for removal of proteins (83). Other size based techniques include size exclusion chromatography which involves differential passage of various vesicles and particles through a gel column and their recovery in different elution fractions (84). SEC allows for pure exosomal isolates with intact physical characteristics (85). The run time is however long and limits its use in multiple biological specimens.

In polymer-based precipitation, a polymer, e.g. polyethylene glycol (PEG) precipitates exosomes in a biofluid and the exosomes recovered by low speed centrifugation to pellet down the precipitate (86).

Immunoaffinity enrichment methods use exosomal membrane markers, typically the tetraspanins CD9, CD63 and CD81, to isolate exosomes from biofluids (87,88). The antibodies are immobilized on varying media including magnetic beads, microfluidic devices and chromatography matrices (89,90).

Newer methods of exosome isolation include contact-free sorting which uses ultrasound waves to exert differential acoustic forces on vesicles based on their size and density (91), microfluidics, which utilizes size dependent position of flow of various vesicles in a biofluid in a channel to isolate exosomes (92) and nanoplasmon-enhanced scatter which uses immobilized exosomal antibodies and gold plated nanoparticles to capture and detect exosomes based on scatter patterns (93). Confirmation of exosome isolation can be done using multiple techniques including electron microscopy which identifies the typical cup shaped morphology and vesicle size of 30-120nm, western blotting for exosome markers such as tetraspanins (CD63/CD9/CD81), ALIX, flotillin, hsp70, TSG101, and also nanoparticle tracking analysis for vesicle size and concentration determination (94).

Exosome Use in DLBCL

Due to their relative abundance, longer half-life, presence in multiple body fluids, and a payload that includes nucleic acids, proteins, and small molecules (95) exosomes are poised to contain biomarkers that may be informative in the prognostication and management of DLBCL patients at multiple stages. The nucleic acid and/or protein cargo in exosomes can be used for molecular analyses in DLBCL. Considering the relative abundance of exosomes in body fluids, especially serum where there is a concentration of about $3x10^6$ exosomes/microliter, this presents a readily available source of biologic material for multiparametric studies leading to more exhaustive characterization of DLBCL types, prognostication, surveillance during and post treatment, and understanding of mechanisms of resistance to therapy. Rutherford et al characterized mutations in cell lines and their exosomes using RNA sequencing and found that nearly one third of mutations in the cell lines were detected in the exosomes (96). This was surprising as exosomes contained a smaller subset of RNAs than the cell lines, a finding that also suggests a likely enrichment of mutated RNA in exosomes.

Exosomes as Prognostic Markers in DLBCL

Evaluation of exosomal nucleic acids can also provide additional prognostic information. Feng et al. found that circulating exosomal expression of miR-99a-5p and miR-125b-5p were significantly higher in chemoresistant vs. chemosensitive DLBCL patients ($p<0.001$) (97). Determination of predictive value of these miRNA using ROC curve analysis showed that the area under the curve (AUC) values of miR-99a-5p, miR-125b-5p, and IPI scores were 0.744 ($p < 0.001$), 0.7802 ($p < 0.001$), and 0.6308 ($p < 0.05$), respectively. The combined predictive value of miR-99a-5p and IPI score, had AUC of 0.8326 ($p < 0.001$) while that of miR-125b-5p combined with IPI score, had an AUC of 0.8143 ($p < 0.001$). This suggests that determination of exosomal expression of miR-99a or miR-125b-5p, when combined with IPI may be a robust way to predict response to therapy. Zare et al. observed a significant increase in exosomal miR-155 levels in refractory/relapsed DLBCL patients compared to responding patients or patients still on R-CHOP therapy (98). They also observed a higher concentration in refractory/relapsed patients.

Exosomes in Post Therapy Surveillance

Exosomes and their associated cargo hold promise in their application as a non-invasive biomarker for DLBCL patients. They could be of great benefit in post treatment surveillance of DLBCL patients as a means to avoid radiation exposure of PET scans and unnecessary testing associated with false-positive PET scans. Di et al identified a panel of five circulating exosomal miRNAs as noninvasive biomarkers of DLBCL consisting of hsa-miR-379-5p, hsa-miR-135a-3p, hsa-miR-4476, hsa-miR-483-3p and hsa-miR-451a (99). hsa-miR-379-5p, hsa-miR-135a-3p, hsa-miR-4476 were overexpressed in DLBCL compared to healthy controls, while hsa-miR-483-3p and hsa-miR-451a were expressed at lower levels. The panel had an AUC of 0.951 (95% CI0.847-0.993) in the testing phase, 0.841 (95% CI, 0.767-0.900) in the validation phase and 0.824 (95% CI, 0.760-0.877) in the combined testing and validation phase.

Exosomes as Mediators of Resistance and Targets for New Therapies

Exosomes play crucial roles in survival of lymphoma cells, evasion of immune response and resistance to therapy. Poggio et al showed that exosomal PD-L1 suppressed T-cell function in lymph nodes draining tumor sites, and promoted tumor growth across different tumor types (100.). Local blockade of exosomal PD-L1, inhibited growth of tumor locally as well as at distant sites injected synchronously or at a later time to the PD-L1 block.

Tumor derived exosomes contain tumor antigens and MHC-I and MHC-II molecules allowing direct presentation and activation of CD8 and CD4 T-cells and cross presentation to T cells via transfer to antigen presenting cells (101,102) . Whether these mechanisms are also operational in DLBCL remains to be determined. Studies of exosomes have provided insights to mechanisms of resistance in DLBCL and possible targets for new therapy. Chen et al showed a dual role of tumor derived exosomes (TEX) in DLBCL (103.). DLBCL exosomes when incubated with dendritic cells (DC) resulted in increased proliferation and activation of dendritic cells. The dendritic cells were able to cause greater T cell expansion after incubation with the exosomes. Tumor derived exosomes however resulted in increased PD-1 expression and increased apoptosis of Th2 cells. They also demonstrated that the TEXs play a role in enhancing cell proliferation, invasion, migration, and angiogenesis with promotion of tumor growth in vivo. Their findings suggest a role for targeting exosome inhibition in developing new therapies for DLBCL or use of exosome derived vaccines to augment the anti-lymphoma immune response.

Koch et al demonstrated the presence of side population (SP) cells in DLBCL, which have stemness properties and are capable of propagating tumor growth (104). They showed an equilibrium of SP and non-SP cells, with no stemness properties, whereby exosome mediated Wnt signaling transformed non-SP cells to SP cells, and vice versa.

Exosomes have been shown to play a role in drug resistance of hematopoietic malignancies to therapy. Koch et al showed that after initial accumulation in the nucleus, the site of action of doxorubicin and pixantrone, these chemotherapeutic agents are exported into exosomes and then released from the cell, leading to reduced

amount of drug at the site of action and development of resistance (105). They showed that inhibition of ABCA3, a protein involved in the transport of these drugs into exosomes resulted in trapping of the drugs in the nucleus and higher sensitivity to doxorubin and pixantrone.

Exosomal CD20 has also been shown to shield DLBCL cells from anti-CD20 immunotherapy providing a mechanism for evading this therapy (106). This mechanism of resistance was again attenuated by inhibition of ABCA3 which is involved in exosome biogenesis. Serial profiling of exosomal nucleic acids and/or proteins during therapy may identify more products involved in resistance and help develop a resistance signature during therapy that necessitates prompt therapy changes. It could also help guide development of new therapeutic targets.

Challenges and Concluding Thoughts

Since exosomes are released by multiple cell types, an obvious challenge in the use of exosomal cargo or liquid biopsy techniques in general as biomarkers of DLBCL is the enrichment of tumor specific exosomes in order to avoid dilution of information in tumor exosomes by exosomes from non-neoplastic tissue. Enrichment for tumor derived exosomes before subsequent analysis of exosomal cargo would address the issue of background noise from non-neoplastic exosomes. Castillo et al identified specific surface proteins on prostate cancer cell lines derived exosomes and used antibodies to these proteins for immunocapture in plasma of prostate cancer patients to enrich for tumor derived exosomes for downstream analysis of the tumor derived exosomal cargo (107). Mizutani et al also demonstrated isolation of prostate cancer specific exosomes using immunocapture with prostate specific antibodies. (108) Similar approaches can be employed as pre-analytical step in studying exosomes in fluids from DLBCL patients.

Another challenge in routine use of exosomes in cancer management is the difference in detected cargo observed using different methods of isolation. Jeppesen et al used high resolution gradient fractionation to separate small extracellular vesicles from non-vesicular proteins and direct immunoaffinity capture to isolate exosomes from other vesicles (109). Analyses of these exosomes showed absence in the cargo of some nucleic acids and proteins hitherto found to be present in exosomes using other methods. Contamination of exosomal isolates by other vesicles and non-vesicular molecules probably accounts for such variation. Guidelines to confirm purity of isolation would need to be developed to prevent inter-laboratory variation of exosomal content analyses.

Exosomes thus have great potential for not only improving diagnosis, prognostication, and monitoring of DLBCL, but provide a means of understanding the biology of DLBCL with the ultimate goal of improving care for patients.

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